

Molecular analysis of regulation of gene expression of the human erythroid anion exchanger (AE) 1

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Abstract The anion exchange protein AE1 is the most abundant membrane protein in human erythrocytes mediating the electro-neutral chloride/bicarbonate exchange. We identified a promoter region in the 5' flanking region of the human AE1 gene which controls transcription in a cell type independent manner. In addition a second, distal promoter element mediates gene expression only in erythroid cells and in dependence upon differentiation. Within this distal promoter region we defined a 44 bp sequence containing a novel CT-rich motif with very strong promoter activity whereas a second 28 bp segment suppresses gene expression.

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Key words: Band 3; Anion exchanger 1; Erythroid specific expression; CT-rich region; Red blood cell maturation

1. Introduction

The anion exchange protein 1 (AE1, band 3) is the major integral membrane protein of erythrocytes. It functions as an electroneutral reversible exchanger of bicarbonate for chloride facilitating removal of carbon dioxide and increasing the carbon dioxide capacity of blood. Additionally, AE1 is thought to be involved in membrane-cytoskeletal interaction and the assembly of the membrane skeleton (for review see [1–3]). AE1 deficient mice [4,5] develop spherocytosis and severe hemolytic anemia probably due to the enhanced removal of abnormally shaped erythrocytes by the spleen.

AE1 belongs to the family of anion exchangers of which at least three members (AE1–3) have been identified by cDNA cloning from different species and tissues (for review see [2,6,7]). The human AE1 cDNA has been cloned from a fetal liver cDNA library [8,9] and has been mapped to chromosome 17 [10]. In chicken erythroid precursor cells four variant AE1 proteins are expressed which are encoded by a total of 14 AE1 transcripts with different translation initiation sites [11]. Twelve of these transcripts are derived from two different erythroid specific promoters P1 and P2 which are located immediately upstream of the transcription start sites. Within these promoter regions the chicken AE1 gene contains typical homologs of presumptive TATA, Sp1, Ap1, Ap2 and CCAAT binding sites [12]. The expression of two further transcripts which are abundant in both chicken erythrocytes and kidney cells is regulated by the promoter P3 [11].

Similar to the chicken gene, AE1 expression in mammals is

not restricted to erythroid cells but is also found in human brain [13]. An amino-terminal truncated version of AE1 exists in renal intercalated cells of the A type. In rat the 5' end of renal AE1 mRNA could be mapped to intron 3 suggesting an alternative promoter within this intron [14]. In human kidney a truncated AE1 primary transcript is alternatively spliced leading to mRNA in which methionine 66 is used as translation initiation codon [15]. It has been shown for the mouse gene that a renal specific promoter located within intron 3 is responsible for the expression of the truncated renal protein and that this promoter is not active in erythroid cells at different stages of erythroid differentiation [16]. Within intron 3 potential TATA sequences located downstream of a cluster of putative transcription factor binding sites could be detected in the mouse gene [17] as well as in the human gene [18]. In contrast to the avian gene such TATA sequences are missing in the 5' flanking region of mammalian AE1 genes [14,17–19]. As expected for a TATA-less promoter the AE1 transcription initiation sites of erythroid AE1 mRNA are heterogeneous and have been mapped in Friend murine erythroleukemic cells to five clustered sites upstream of the translation initiation codon [19]. A basal promoter activity of a 135 bp region immediately 5' flanking the mouse AE1 gene could be detected in erythroid and non-erythroid cells [16]. An additional 200 nucleotides were required to obtain induced promoter activity in differentiating erythroid cells.

As a first approach towards understanding the molecular regulation of the erythroid expression of the human AE1 gene, we isolated the 5' flanking region and monitored promoter activity of different fragments by reporter gene expression. In addition to a cell type independent proximal promoter, we detected a second promoter region, located more upstream, which confers cell type and differentiation specific gene expression. Within this region we defined a CT-rich sequence with strong promoter activity as well as putative repressor protein binding sites.

2. Materials and methods

2.1. Isolation of the human AE1 gene 5' flanking sequence and construction of reporter plasmids

A human genomic cosmid library cloned in pcos2EMBL was screened with the complete α -³²P-labeled AE1 cDNA. Positive clones were characterized by restriction mapping and subsequent Southern blot hybridization. A 5.5 kb fragment representing the 5' flanking region of the human AE1 gene was identified by oligonucleotide hybridization (position –100 to –80 relative to the translation initiation site). 1787 bp 5' to the translation initiation site were sequenced on both strands using α -³²S-dATP [20] and have been deposited in GenBank (accession number AJ011714). Appropriate restriction and PCR amplified fragments as well as fragments derived from exonuclease digestion of longer sequences were subcloned in front of the luciferase gene in the pGL2-Basic reporter plasmid (Promega, Fig. 2,5,6).

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2.2. Cell lines and culture

K562 cells (ATCC CCL 243) were grown in RPMI 1640 supplemented with 10% fetal calf serum (Life Technologies), 2 mM L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin at 37°C and 5.5% carbon dioxide. Hemin was added for induction of erythroid differentiation at a final concentration of 35.5 µM. HeLa (ATCC CCL 2) cells were grown in minimal essential medium containing 10% calf serum, L-glutamine (2 mM), penicillin (50 U/ml) and streptomycin (50 µg/ml).

2.3. Quantification of AE1 transcription and hemoglobin synthesis

AE1 transcripts were quantified by reversed transcription based polymerase chain reaction (RT-PCR) in combination with temperature gradient gel electrophoresis (TGGE) [21]. As an internal reference for reverse transcription we used mutated AE1 copy RNA (AAG→GCA, Lys⁵³⁹Ala). 4 µg K562 total RNA, prepared at different times after hemin treatment, was mixed with 10⁶ copies of the corresponding mutated copy RNA and were reverse transcribed using primer oko10: 5' GCG GAT CCC TTC TGT AGT GGG TGG TCC 3'. Following ultrafiltration (Centricon 100, Amicon), 1/10 of the cDNA was used to amplify a 399 bp AE1 fragment with primers oko9 (5' CGG GGA TCC GCT GGT GTT GTT TGA GGA AGC C 3') and oko10. As a standard for TGGE, the corresponding AE1 fragment subcloned in pUC18 was excised and labeled with α³²P]dCTP using Klenow polymerase. One tenth of the PCR reaction was mixed with 20000 cpm of the standard fragment. After denaturation (5 min, 94°C) and renaturation (30 min, 60°C) homo- and heteroduplexes were formed and separated by parallel TGGE in a 8% urea/5% polyacrylamide gel and a linear 20–60°C temperature gradient for 30 min. Homo- and heteroduplex DNA was detected by autoradiography and quantified by liquid scintillation counting. Hemoglobin of K562 cells was quantified as methemoglobin cyanide (Merck).

2.4. Transfection and reporter gene analysis

CsCl purified recombinant plasmids were introduced in K562 cells in complex with cationic liposomes (Lipofectin, Life Technologies). Transfections of differentiating K562 cells were performed by addition of 35.5 µM hemin to the medium 36 h prior to transfection. 20 µg reporter gene plasmid was cotransfected with 10 µg pCMV-β-gal as an internal reference containing the prokaryotic β-galactosidase under the control of the cytomegalovirus promoter. 3×10⁶ cells were washed twice in serum free medium and resuspended in 3 ml medium containing 30 µg lipofectin and 30 µg plasmid DNA. Cells were incubated for 6 h before addition of 6 ml of supplemented RPMI medium. Cells were harvested 48 h post transfection when luciferase activity was maximal. HeLa cells were transfected by calcium phosphate precipitation [22]. Luciferase activity was determined with the luciferase assay system (Promega). Transfection efficiency was standardized by measuring β-galactosidase activity using a chemiluminescent assay (Tropix). Luciferase activity was referred to the protein level of the extracts and normalized by the exogenous galactosidase activity.

2.5. Nuclear extract preparation

From untreated K562 cells and from cells that were previously stimulated to differentiation with hemin for 36 h nuclear proteins were extracted and stored according to Osborn et al. [23]. Extracts were successively purified and concentrated under non-denaturing conditions by ultrafiltration using filter units with molecular cutoffs of 100, 30 and 10 kDa (Amicon). The protein concentration was determined using bicinchoninic acid (Pierce). The fraction containing proteins and protein complexes with a molecular mass >100 000 was used in DNase I footprint experiments and in gel mobility shift assays unless stated otherwise. In some experiments we used nuclear extracts filtered solely through a membrane with a molecular cutoff of 10 kDa.

2.6. DNase I footprinting

HindIII/KpnI fragments originating from different recombinant pGL-Basic clones were selectively radiolabeled with Klenow polymerase at the HindIII site and were used as a probe in DNase I footprint experiments as described [24]. 15 fmol labeled DNA and 1.5 µg poly-[dI-dC]poly[dI-dC] were incubated at 25°C in the absence and presence of 5 µg nuclear proteins isolated from hemin stimulated cells. 0, 0.625 and 1.25 ng DNase I were added for 2 min before the DNase

was inactivated by addition of EDTA, SDS and proteinase K followed by phenol extraction and ethanol precipitation. The reaction products were analyzed on a denaturing polyacrylamide gel. As reference we took reaction products obtained by Sanger sequencing with primers of which the 5' end matched to the labeled end of the investigated fragment.

2.7. Band shift assay

0.5–5 fmol end labeled DNA was purified by gel electrophoresis and incubated with different amounts of poly[dI-dC]poly[dI-dC] in 42 mM NaCl, 20 mM KCl, 20 mM N-(2-hydroxyethyl)piperazine-N⁹-2-ethanesulfonic acid (pH 7.9), 10 mM MgCl₂, 5 mM CaCl₂, 0.1 mM EDTA, 0.25 mM phenylmethylsulfonyl fluoride and 2.5 mM dithiothreitol. 5 µg of nuclear proteins were added and the mixture was incubated for 30 min at room temperature. The reaction mixtures were applied on a 5% polyacrylamide (29:1) gel and separated at 4°C and 4 V/cm as described [25]. For the analysis of double stranded oligonucleotides, 0.5 pmol of complementary single stranded oligonucleotides (Fig. 3c) were denatured in 20 µl 50 mM Tris (pH 7.5), 10 mM MgCl₂, 1 mM dithiothreitol and 50 µg/ml BSA for 15 min at 80°C and annealed at room temperature overnight followed by radioactive labeling of specific nucleotide overhangs using Klenow polymerase.

3. Results

3.1. Two regulatory regions with different cell type specificity

To identify sequences responsible for AE1 expression, we cloned a 5.5 kb DNA fragment encoding the 5' flanking region of the human AE1 gene. Comparison of 1787 bp 5' to the translation initiation site (excluding the intron 1 sequence at position –69) and 1710 bp of the mouse gene [19] revealed conspicuous homology of the nucleotides –170 to –380 and an accumulation of transcription factor binding sites within the same region of both genes (data not shown) suggesting a similar localization of the human and the mouse AE1 promoter. In addition, we found another cluster of recognition sites for transcription factors in the human gene between nucleotides –1300 to –1700 which is missing in the mouse AE1 5' flanking region. To investigate the function of the putative regulatory regions, we dissected the 5.5 kb fragment into three parts which were cloned upstream of the luciferase reporter gene (Fig. 1a). Luciferase activities of the recombinant constructs pGL-A, pGL-B and pGL-C were analyzed after transfection of erythroleukemic K562 cells or epithelial HeLa cells as a non-erythroid reference and compared with a control plasmid containing the SV40 promoter (pGL-Control).

An enhanced luciferase activity was measured in transfections using the recombinant plasmid pGL-C indicating that the AE1 promoter is located within this 1041 bp fragment. This corresponds to the promoter localization directly 5' to the transcription initiation sites reported for the mouse AE1 gene [16]. Reporter gene expression was similar in both K562 and HeLa cells showing a cell type independent promoter activity. In addition, we found that a more distantly located region B comprising a second cluster of transcription factor binding sites (pGL-B) mediates gene expression independently of the proximal AE1 promoter. Plasmid pGL-B led to a significant increase of reporter gene expression in K562, but in contrast to expression levels obtained with the proximal promoter (pGL-C), pGL-B did not mediate gene expression in HeLa cells. This indicates a cell type specific activity of this additional, independent promoter element. We further dissected fragment B into two parts B1 (–1787 to –1497) and B2 (–1496 to –1122) and compared their potency upon gene expression to the other fragments. Subsequent transfection

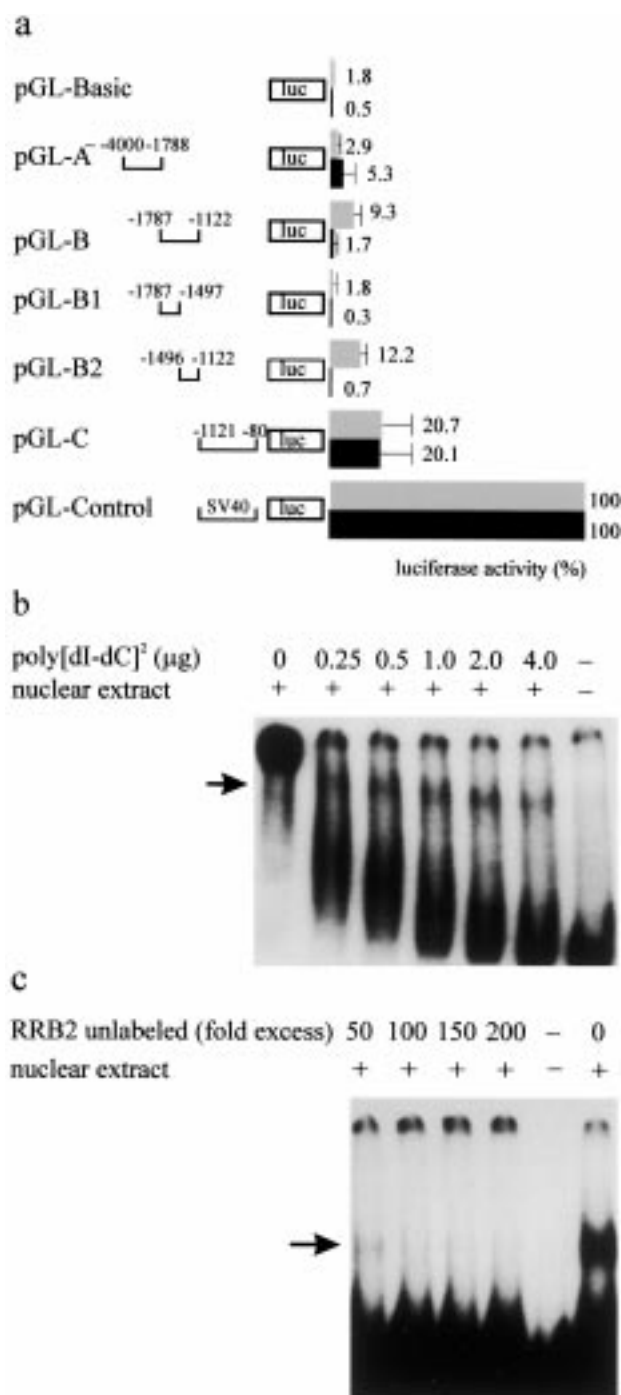


Fig. 1. Independent AE1 promoter elements with different cell type specificity. a: Luciferase activity in cell extracts obtained 48 h post transfection of K562 cells (gray columns) and HeLa cells (black columns) with different recombinant reporter plasmids referred to SV40 promoter mediated luciferase expression (pGL-Control, 100%). The length of the inserted fragments and distances to the AE1 translation start excluding intron 1 is indicated. Values represent mean \pm S.D. of three independent experiments ($n=5$). b: Band shift assay of 1 ng α - 32 P-labeled RRB2 fragment in the presence (+) or absence (–) of 5 μ g nuclear extract (molecular mass $>10\,000$) and in the presence of increasing amounts (0–4 μ g) of poly[dI-dC]. c: Competition of specific interaction of RRB2 to nuclear proteins in a band shift assay by addition of an increasing amounts of unlabeled RRB2 fragment (0–200-fold excess) in the presence of 2 μ g poly[dI-dC]. Specific DNA/protein complexes are marked by arrows.

experiments identified fragment B2 as functionally active with a relative expression rate of 12.2%. Based on these results, we designated fragment B2 the regulatory region B2 (RRB2).

RRB2 possesses an independent regulatory activity which should be due to protein binding of specific transcription factors within RRB2. Therefore, we performed band shift experiments using the radio-labeled RRB2 fragment. As shown in Fig. 1, K562 nuclear proteins bind indeed specifically within RRB2 as seen by the formation of shifting DNA/protein complexes in the presence of increasing amounts of unspecific competitor DNA (Fig. 1b) and as seen by the competition of such complexes by the addition of a 50–100-fold excess of unlabeled RRB2 fragment (Fig. 1c).

3.2. Changes of promoter activity during erythroid differentiation

In a next series of experiments, we looked for the potential role of the different AE1 promoter regions upon enhanced AE1 expression during red blood cell maturation. We investigated changes of the AE1 promoter activity during erythroid differentiation by quantification of AE1 gene transcription in K562 cells which had been induced to differentiate with hemin (Fig. 2a). In parallel we measured the production of hemoglobin serving as a marker of differentiation [26]. Upon treatment of K562 cells with hemin, hemoglobin increased more than 2.5-fold from an initial value of 1.3 pg/cell to a maximum of 3.4 pg/cell after 96 h and decreased slightly after 5 days. In parallel to the proceeding erythroid differentiation the AE1 mRNA level rose about 2-fold from three copies/cell to six copies/cell (100%) after 36 h and decreased after 72 h to basal transcription indicating that enhanced AE1 promoter activity is linked to red blood cell maturation.

Therefore, we examined changes of AE1 promoter activity during erythroid differentiation by transfection of reporter plasmids in non-stimulated K562 cells and cells which were stimulated to differentiation with hemin for 36 h (Fig. 2b). Treatment of the cells with hemin significantly decreased SV40 promoter dependent expression of the reporter gene making the pGL-Control plasmid unsuitable as a reference for this study (data not shown). However, we detected increased luciferase activity in hemin stimulated cells when AE1 promoter constructs (pGL-B, pGL-C) were transfected, indicating that the investigated 5' flanking sequences control genes expression in dependence of differentiation. This effect was most significant after transfection of pGL-B with a more than 3-fold enhancement of luciferase activity in contrast to pGL-C transfections which showed only a slight increase of the basal gene expression. Thus, region B contains not only a cell specific but also a differentiation dependent promoter element RRB2.

To further support this finding, we investigated if this differentiation specific enhancement is reflected by changes of the interaction properties of AE1 promoter sequences to nuclear proteins. Nuclear extracts from undifferentiated and from hemin stimulated K562 cells were each filtered through membranes with different molecular cutoffs to pool proteins with similar molecular mass which were subsequently analyzed for their interaction with RRB2 in band shift assays. As shown in Fig. 2c, we found RRB2 binding proteins enriched in fractions of proteins or protein complexes with a molecular mass higher than 100 000. Three different bands could be detected leading to the conclusion that several sites of protein interaction exist

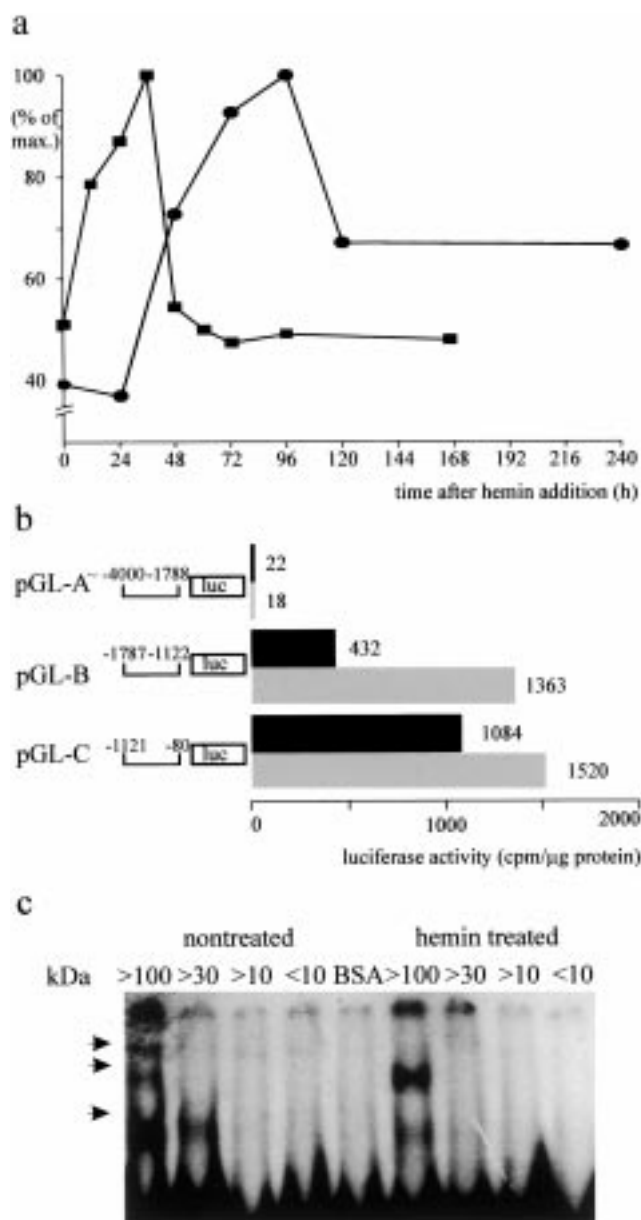


Fig. 2. Enhancement of AE1 expression and AE1 promoter activity during erythroid differentiation. a: Time course of AE1 mRNA (squares) and hemoglobin (circles) formation following induction of K562 cell differentiation with hemin. All values are given as percentage of maximum values (AE1 mRNA 6.05 copies/cell; hemoglobin 3.4 pg/cell) as mean of two independent experiments ($n=3$). b: Reporter gene expression mediated by AE1 5' flanking fragments in extracts of untreated (black columns) and 36 h hemin treated (gray columns) K562 cells. The length of the fragments and their relative position to the AE1 translation start site excluding intron 1 is indicated by numbers. Values represent mean of two experiments ($n=5$). c: Gel retardation assay of 1 ng labeled RRB2 fragment in the presence of 2 μg nuclear proteins/protein complexes of different molecular mass obtained from untreated and 36 h hemin treated K562 cells and in the presence of 2 μg poly[dI-dC]poly[dI-dC]. In a control reaction, nuclear proteins were substituted by 10 μg bovine serum albumin (BSA). Specific DNA/protein complexes are indicated by arrows.

in RRB2. Remarkably, we detected changes in the band pattern using nuclear extracts from hemin treated cells indicating that the assembly of RRB2/protein complexes might be changed upon erythroid differentiation and probably reflect-

ing enhanced promoter activity during red blood cell maturation.

3.3. Elements enhancing and silencing gene expression are located within RRB2

To define distinct base pairs within RRB2 which contribute to AE1 transcription, we first dissected this region into five smaller parts B3–B7 (Fig. 3a) and analyzed their promoter activity upon luciferase expression. 122 bp in the upstream part of RRB2 had no promoter activity (pGL-B7) but removal of this sequence (pGL-B4) resulted in a significant enhancement of luciferase activity which was even higher than SV40 driven reporter gene expression. The absence of nucleotides –1374 to –1331 decreased luciferase activity to background levels (pGL-B5) indicating that this 44 bp stretch is responsible for a strong expression of genes located downstream. We defined this 44 bp sequence as a distal activator segment (DAS). The upstream nucleotides –1375 to –1402 of DAS reduced activity (pGL-B3) to similar levels as detected in case of RRB2 (pGL-B2) leading to the conclusion that a distal silencer segment (DSS) is located within this 28 bp region. Removal of the downstream part of RRB2 (nucleotides –1122 to –1285, pGL-B6) led to enhanced luciferase activity suggesting the existence of additional silencing elements within this region. Inversion of the orientation of RRB2 (pGL-B2rev) or fragment B6 (pGL-B6rev) in relation to the luciferase gene resulted in a 2–3-fold reduction but not in a complete absence of transcription activity indicating that RRB2 function is dependent upon its orientation.

Based on the results of the reporter gene analysis, it can be expected that DAS and DSS contain transcription factor binding sites for activator and suppressor proteins. We tested this idea by footprint analysis of RRB2 (Fig. 3b) and band shift assays (Fig. 3c). Indeed, we detected two footprints within DAS corresponding to nucleotides –1353 to –1343 (TTTCTCTCTC) and to nucleotides –1365 to –1362 (GTGT) indicating that these novel protein binding sites interact with sequence specific transcription factors responsible for AE1 transcription mediated by DAS. The motif TTTCTCTCTC is unique in the 5' flanking region of the AE1 gene and consists exclusively of pyrimidines conferring an extreme purine/pyrimidine asymmetry to the double stranded DNA.

In addition, three protein binding motifs were present within DSS. For one of them nucleotides involved in protein binding were slightly different on the coding and non-coding strand probably reflecting asymmetrical DNA binding. The other two motifs were defined as CATAAAC (nucleotides –1392 to –1386) and as GATTCTGCCT (nucleotides –1383 to –1374). It seems likely that these motifs bind to repressor proteins.

We confirmed protein binding to DAS and DSS by band shift experiments (Fig. 3c) using double stranded oligonucleotides corresponding in their sequence to the footprint areas within DAS (oligoDAS) and DSS (oligoDSS). Investigation of oligoDSS revealed three bands corresponding to DNA/protein complexes indicating that different kinds of proteins bind to this oligonucleotide. Similarly, investigation of oligoDAS led to the appearance of three bands in the shift experiment. In one case, DNA/protein binding was stable even in the presence of the highest amounts of competitor used, showing that protein binding within DAS is stronger than in DSS. We

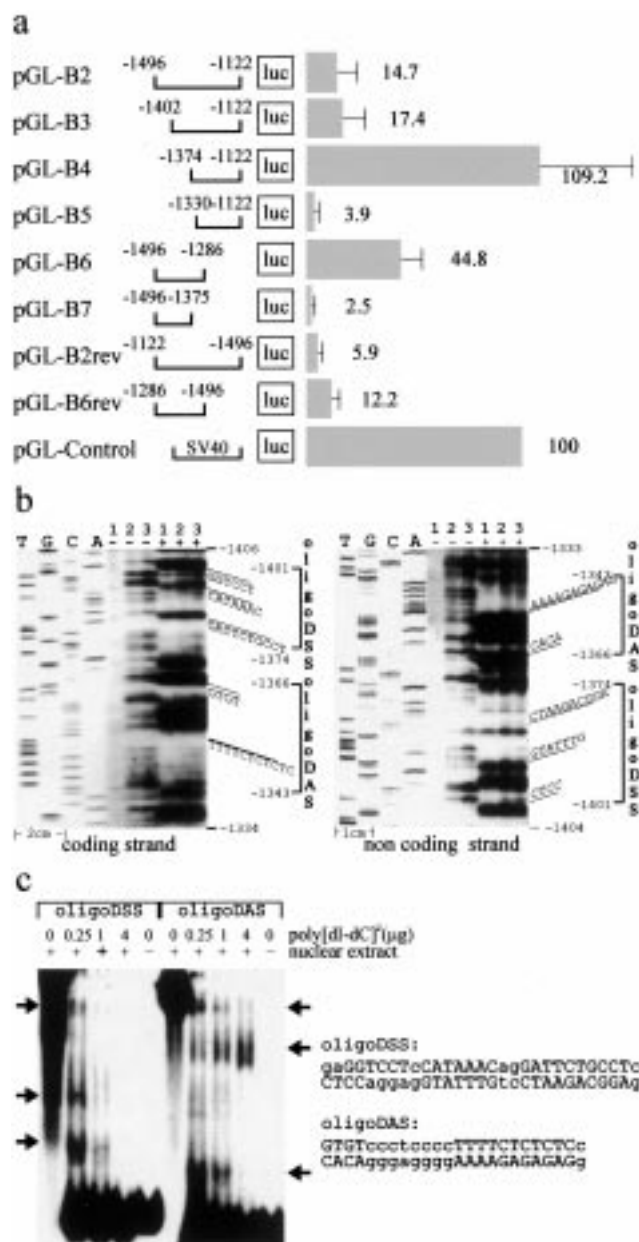


Fig. 3. Enhancer and silencer elements located within the distal promoter region RRB2. a: Luciferase activity of K562 cell extracts obtained after transient expression of reporter plasmids containing different fragments of RRB2 (pGL-B2) and referred to SV40 promoter activity (pGL-Control, 100%). The length of the analyzed fragments and their relative position to the AE1 translation start site excluding intron 1 are indicated. 'Rev' indicates an inverted orientation of the fragment relative to the reporter gene. Values represent means \pm S.D. ($n=3$) of three independent experiments. b: DNase I footprint analysis of the regulatory region B2. Digestion was performed with 0 (1), 0.625 (2) and 1.25 (3) ng DNase I in the absence (-) or presence (+) of nuclear proteins. Even in the absence of DNase I (lane 1), DNA was hydrolysed when nuclear proteins were present (+) indicating endogenous DNase activity of the nuclear extract. Reference lanes of sequencing reactions indicate the positions of the footprints relative to the AE1 translation initiation site excluding intron 1. Brackets with numbers indicate footprint regions of which oligonucleotides were deduced used in band shift assays (c). c: Gel retardation assay of oligonucleotides corresponding to footprint areas. Capital letters in the nucleotide sequence correspond to the footprinted nucleotides. 10 pg (0.5 pmol) of double stranded oligonucleotides was incubated in the presence (+) or absence (-) of K562 nuclear proteins with increasing amounts of poly[dI-dC]-poly[dI-dC]. Shifted DNA/protein complexes are indicated by arrows.

conclude that DAS and DSS are *cis*-acting binding sites for transcription factors which may be involved in tissue and differentiation dependent regulation of the AE1 gene.

4. Discussion

Sequence analysis of the 5' flanking region of the human and the mouse AE1 gene shows striking sequence similarity about 400 nucleotides upstream of the translation initiation site. Within this region multiple putative transcription factor binding sites are located [17,18] and transcription is initiated at different start sites in the rat and mouse genes [14,19]. In this study we provide evidence that the promoter of the human AE1 gene is in a similar location as in the rat and mouse genes. This proximal promoter regulates expression in a cell type independent manner since it is active in epithelial cells which were not expected to express AE1. This result corre-

sponds to that obtained for the mouse AE1 promoter [16] which is active in Rauscher erythroleukemic cells as well as in fibroblasts (NIH3T3). We conclude that additional regulatory sequences exist which might confer cell type specific AE1 expression in erythroid cells. Similar to the chicken AE1 gene, we could show that a second promoter exists which is located far upstream of the proximal promoter. Within this distal element, called RRB2, a similar accumulation of putative transcription factor binding sites is located. In contrast to the proximal promoter, RRB2 is active only in K562 cells indicating that RRB2 activity might be restricted to erythroid cells and may reflect its cell type specificity. Although RRB2 is able to mediate reporter gene expression *in vitro*, its function and cooperation with the downstream promoter *in vivo* is elusive. Further studies will show, if both regulatory regions are active *in vivo* and how they might work together.

To investigate a possible role for regulatory elements during erythroid differentiation, we chose the human cell line K562. These cells transcribe the AE1 gene in dependence upon the erythroid differentiation suggesting a coordinated regulatory mechanism. Both regulatory elements are more active in differentiating cells but the effect is more significant with the distal promoter RRB2. It seems possible that erythroid specific transcription factors like GATA and NF-E2 contribute to the enhanced AE1 expression during red blood cell maturation since GATA binding sites are located within both regulatory regions. Those factors may coordinate expression of several genes like AE1 and hemoglobin during red blood cell maturation [27–29].

A closer examination of the distal element RRB2 led to the detection of a 44 bp region which serves as a promoter in reporter gene assays even stronger as the viral SV40 promoter. This DAS is characterized by strong interaction to nuclear proteins and the existence of a purine/pyrimidine asymmetry within an 18 bp stretch consisting exclusively of pyrimidines. This novel CT-rich motif is unique in the 5' flanking region of the human AE1 gene and might be involved in the formation of an intramolecular triplex/single stranded DNA conformer called H-DNA [30]. H-DNA conformers are abundant in DNase I hypersensitive sites and might break chromatin struc-

tures to facilitate transcription. For the *c-myc* gene it has been shown that a CT-rich motif is responsible for approximately 80% of the promoter activity [31]. In addition to sequences in DAS, we could define DSSs in the neighborhood of DAS which reduce promoter activity of RRB2.

The identification of the protein binding motifs DSS and DAS provides the molecular basis for the identification of transcription factors involved in regulation of AE1 gene expression. A closer examination of the tissue specificity of DAS will reveal if this element may serve as a tissue specific promoter in gene targeting experiments or in gene therapy.

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References

- [1] Jay, D.G. (1996) *Cell* 86, 853–854.
- [2] Tanner, M.J.A. (1993) *Semin. Hematol.* 30, 34–57.
- [3] Passow, H. (1986) *Rev. Physiol. Biochem. Pharmacol.* 103, 61–203.
- [4] Peters, L.L., Shivdasani, R.A., Liu, S.-C.L., Hanspal, H., John, K.M., Gonzalez, J.M., Brugnara, C., Gwynn, B., Mohandas, N., Alper, S.L., Orkin, S.H. and Lux, S.E. (1996) *Cell* 86, 917–927.
- [5] Southgate, C.D., Chishti, A.H., Mitchell, B., Yi, S.J. and Palek, J. (1996) *Nature Genet.* 14, 227–230.
- [6] Alper, S.L. (1991) *Annu. Rev. Physiol.* 53, 549–564.
- [7] Kopito, R.R. (1990) *Int. Rev. Cytol.* 123, 177–199.
- [8] Tanner, M.J.A., Martin, P.G. and High, S. (1988) *Biochem. J.* 256, 703–712.
- [9] Lux, S.E., John, K.M., Kopito, K.M., Kopito, R.R. and Lodish, H.F. (1989) *Proc. Natl. Acad. Sci. USA* 86, 9089–9093.
- [10] Showe, L.C., Balantine, M. and Huebner, K. (1987) *Genomics* 1, 71–86.
- [11] Cox, K.H., Adair-Kirk, T.L. and Cox, J.V. (1995) *J. Biol. Chem.* 270, 19752–19760.
- [12] Kim, H.-R.C., Kennedy, B.S. and Engel, J.D. (1989) *Mol. Cell. Biol.* 9, 5198–5206.
- [13] Havenga, M.J.E., Bosman, G.J.C.G.M., Appelhans, H. and De Grip, W.J. (1994) *Mol. Brain Res.* 25, 97–104.
- [14] Kudrycki, K.E. and Shull, G.E. (1993) *Am. J. Physiol.* 264, F540–F547.
- [15] Kollert-Jöns, A., Wagner, S., Hübner, S., Appelhans, H. and Drenckhahn, D. (1993) *Am. J. Physiol.* 265, F813–F821.
- [16] Sahr, K.E., Daniels, B.P. and Hanspal, M. (1996) *Blood* 88, 4500–4509.
- [17] Sahr, K.E., Taylor, W.M., Daniels, B.P., Rubin, H.L. and Jarolim, P. (1994) *Genomics* 24, 491–501.
- [18] Schofield, A.E., Martin, P.G., Spillett, D. and Tanner, M.J.A. (1994) *Blood* 84, 2000–2012.
- [19] Kopito, R.R., Andersson, M.A. and Lodish, H.F. (1987) *Proc. Natl. Acad. Sci. USA* 84, 7149–7153.
- [20] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA* 74, 5463–5467.
- [21] Riesner, D., Steger, G., Wiese, U., Wulfert, M., Heibey, M. and Henco, K. (1992) *Electrophoresis* 13, 632–636.
- [22] Chen, C. and Okayama, H. (1987) *Mol. Cell. Biol.* 7, 2745–2752.
- [23] Osborn, L., Kunkel, S. and Nabel, G.J. (1989) *Proc. Natl. Acad. Sci. USA* 86, 2336–2340.
- [24] Von der Ahe, D. (1991) in: *A Laboratory Guide to In Vitro Studies of Protein-DNA Interactions* (Jost, J.P. and Saluz, H.P., Eds.), *BioMethods*, Vol. 5, pp. 19–54, Birkhäuser, Basel.
- [25] Singh, H., Sen, R., Baltimore, D. and Sharp, P.A. (1986) *Nature* 319, 154–158.
- [26] Rutherford, T.R., Clegg, J.B. and Weatherall, D.J. (1979) *Nature* 280, 164–165.
- [27] Engel, J.D., George, K.M., Ko, L.J., Kornhauser, J.M., Leonard, M.W., Ting, P. and Yamamoto, M. (1991) *Semin. Hematol.* 28, 158–169.
- [28] Orkin, S.H. (1992) *Blood* 80, 575–581.
- [29] Andrews, N.C., Erdjument-Bromage, H., Davidson, M.B., Tempst, P. and Orkin, S.H. (1993) *Nature* 362, 722–728.
- [30] Kolluri, R., Torrey, T.A. and Kinniburgh, A.J. (1992) *Nucleic Acids Res.* 20, 1111–1116.
- [31] Davis, T.L., Firulli, A.B. and Kinniburgh, A.J. (1989) *Proc. Natl. Acad. Sci. USA* 86, 9682–9686.